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Award Number: W81XWH-08-1-0092

TITLE: To Degrade or Not Degrade: Targeting the Androgen Receptor in AR-Dependent Prostate Cancer

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REPORT DATE: April 2012

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-04-2012	Final	3 MAR 2008 - 2 MAR 2012

4. TITLE AND SUBTITLE
To Degrade or Not Degrade: Targeting the Androgen Receptor in AR-Dependent Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-08-1-0092

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Baylor College of Medicine
Houston, TX 77030

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The development and growth of prostate cancer depends on androgens that activate the androgen receptor (AR) in a hormone-dependent manner. The research objective is to develop novel anti-cancer drugs based on PROteolysis TArgeting Chimeric molecules (PROTAC), which enhance AR ubiquitination and degradation by the 26S proteasome. During the course of this project, we have used peptide array technology to screen miniaturized libraries of overlapping peptides using the human E3 ubiquitin ligase, C-terminus of Hsc70-Interacting Protein (CHIP), as probe. We have identified a high-affinity CHIP-binding peptide featuring an Arg-Lys-Xxx-Lys-Lys motif, which is found in all steroid hormone receptors, and which was used to synthesize our first AR-targeting PROTAC. We have reconstituted an in vitro ubiquitination assay using highly purified components, which was used, together with a cell based assay, to evaluate our PROTAC.

15. SUBJECT TERMS
Androgen receptor; peptide array; degron; E3 ubiquitin ligase

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	9	**19b. TELEPHONE NUMBER** *(include area code)*

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INTRODUCTION

Prostate cancer is the most frequently diagnosed malignancy, and the second leading cause of cancer-related deaths in American men. The development and growth of this disease depends on androgens that activate the androgen receptor (AR) in a hormone-dependent manner. While most tumors regress after surgical or chemical ablation therapy, the tumors eventually recur and ultimately cause death. Hence, there is a pressing need to develop new anti-cancer drugs that target AR in the clinically advanced stages of prostate cancer.

Human AR is a member of the steroid hormone receptor family of transcription factors. In addition to AR, other members of this family include the receptors for estrogen (ER), progesterone (PR), glucocorticoid (GR), and mineralocorticoid (MR). It is widely appreciated that receptor availability in cells is regulated by the ubiquitin-proteasome pathway, which targets proteins for destruction by the 26S proteasome. To be degraded, a protein must be recognized by an E3 ubiquitin ligase that differentiates among proteins by binding to one or more specific motifs present in the substrate. These motifs are known as degrons and function as proteolysis targeting signal. However, despite the wealth of biochemical, structural, and cell biological information, it remains largely unknown what degrons are recognized by different E3s, and how protein degradation is controlled in a temporal and spatial manner.

The broad and long-term objective of this research is to develop novel anti-cancer drugs based on *PRO*teolysis *T*argeting *C*himeric molecules (PROTAC). PROTACs are small, hetero-bifunctional molecules consisting of a degron peptide that is covalently linked to an androgen or derivative thereof. While PROTACs have been shown to function in vitro (1), it remains unclear what is the degron of choice and what E3 ligase should be recruited.

BODY

Task 1: To identify AR-derived peptides that are recognized and bound by single-subunit E3s (Months 1-18).

Task 1 was concerned with identifying CHIP-binding motifs by screening miniaturized libraries of synthetic, overlapping 12mer peptides derived from the amino acid sequence of the human receptors for androgen (AR), progesterone (PR), and mineralocorticoid (MR). By screening a MR-derived peptide library, we identified a CHIP-binding peptide that contained an Arg-Lys-Xxx-Lys-Lys (where Xxx is any amino acid) sequence motif. Subsequent bioinformatic analysis showed that a similar motif is found in all steroid hormone receptors and, therefore, may function as a universal CHIP-binding motif (c.f. revised first annual report, Figs. 4 and 5; and third annual report, Fig. 1).

Task 2: To identify "universal" AR-peptides that are recognized by multiple distinct, AR-specific E3s (Months 12-30).

Due to the complex nature of the E3-substrate relationship, we focused our investigation on human CHIP, an E3 ubiquitin ligase that is known to ubiquitinate diverse steroid hormone receptor either alone or in conjunction with Hsp70. The latter is an ATP-dependent molecular chaperone required for the folding of AR and other steroid hormone receptors. However, to develop a CHIP-dependent PROTAC, it was necessary to establish that our bacterially expressed, recombinant human CHIP is functionally active. To do so, we developed an in vitro self-ubiquitination assay using commercially available Ube1 (E1), purified human UbcH5c (E2) and bacterially expressed and purified human CHIP (E3), which functioned both as E3 ubiquitin ligase and substrate in the assay. Since protein ubiquitination is ATP dependent, CHIP self-ubiquitination (as opposed to ubiquitin binding) was easily distinguished by performing the assay in the presence and absence of ATP. As reported in our second annual report, we found that our bacterially expressed, recombinant human CHIP is functional and facilitated CHIP self-ubiquitination in an ATP-dependent manner (c.f. revised second annual report, Fig. 3).

Next, we determined whether human CHIP could also ubiquitinate CHIP substrates, such as human Hsp70. As previously reported, we demonstrated that CHIP ubiquitinated human Hsp70, which resulted in a protein “ladder” consisting of distinct ubiquitinated Hsp70 species (c.f. third annual report, Fig. 3).

Task 3: To synthesize bicalutamide-based PROTACs harboring a universal E3-binding motif and to evaluate their bioactivity in vitro and in living cells (Months 24-36 and period under NCE).

We previously reported the identification and characterization of a MR-derived CHIP-binding peptide (c.f. third annual report). A fluorescein-labeled peptide with sequence Gly-Gly-Ala-Arg-Lys-Ser-Lys-Lys-Leu-Gly-Lys-Leu-Lys) bound full-length CHIP with submicromolar affinity as determined using fluorescence polarization (c.f. third annual report, Fig. 2). The Arg-Lys-Xxx-Lys-Lys containing peptide was also used for the synthesis of our first dihydrotestosterone (DHT)-based AR-targeting PROTAC (c.f. third annual report, Fig. 4). PROTAC synthesis was done by Prof. Kyung-Bo Kim, our collaborator and sub-awardee of this Idea Development Award.

Under the no cost extension, we evaluated our AR-targeting PROTAC. Unfortunately, experiments performed using our life cell assay were inconclusive due to an unresponsiveness of AR in LNCaP cells. This appeared to be an AR-specific problem (K.B. Kim, personal communication) as targeting ER in ER-positive cancer cells was not subject to this technical issue (e.g. Ref. 2). However, developing an ER-targeting PROTAC was not previously approved in the SOW and, therefore, not pursued.

Similarly, we hit a roadblock with full-length AR using our in vitro ubiquitination assay. Since full-length AR cannot be made in functionally active form in bacteria, we purchased AR from a commercial source (Abcam). However, unlike our ubiquitination assay using purified Hsp70 as substrate, ubiquitination of human AR resulted in a protein "smear", which is indicative of poly-ubiquitination (Figure 1). While AR appears to be ubiquitinated in a CHIP-dependent manner (Figure 1, compare lane 1 with lanes 2 and 4), the inability to distinguish between different ubiquitinated AR species made it impossible to interpret our findings. Similar results were also obtained in the presence of Hsp70 that is required for AR folding and is believed to assist in AR ubiquitination (Figure 1, compare lane 1 with lanes 3 and 5). However, despite extensive efforts, we could not further improve our in vitro assay, and only "smeared" bands were observed. We therefore concluded that our assay lacked the sensitivity to determine whether our AR-targeting PROTAC can differentially affect AR ubiquitination.

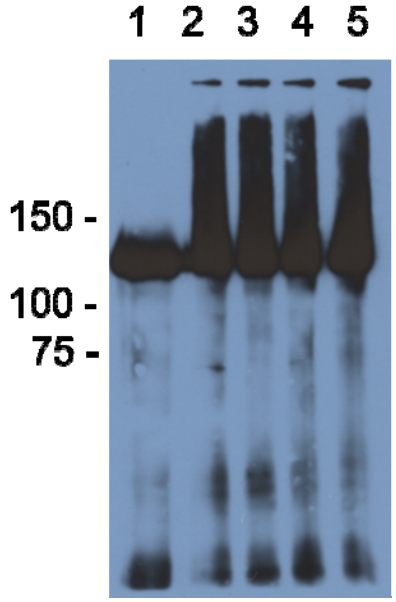

Figure 1: Western blot detection of full-length human AR (Lane 1) and ubiquitinated AR either in the presence of 1 μM DHT (lanes 2 and 4) or 1 μM purified Hsp70 (lanes 3 and 5). Reactions were performed for 2 hours at 30 °C in 50 mM Tris.HCl pH 7.5, 5 mM KCl, 5 mM $MgCl_2$, 5 mM ATP with 1 mM ubiquitin, 0.2 μM E1 (UBE1, Boston Biochem), 50 μM E2 (UbcH5c), 3.5 μM E3 (CHIP), 1 μM full-length AR (Abcam), and either 10% (lanes 2 and 3) or 15% glycerol (lanes 4 and 5). Reactions were stopped by the addition of SDS sample buffer and analyzed by SDS_PAGE on an 8% acrylamide gel. AR was detected by immunoblotting. The position and MW (kDa) of marker proteins are indicated.

KEY RESEARCH ACCOMPLISHMENTS

FY01

- Identified a CHIP-binding peptide using peptide array technology, which is present in all steroid hormone receptors and, therefore, may function as a universal CHIP-binding motif (Task 1.2).
- The Arg-Lys-Xxx-Lys-Lys motif containing CHIP-binding peptide is soluble in aqueous solution, which is a pre-requisite for PROTAC design (Task 2.2).

FY02

- Obtained crystals of a human CHIP-Hsp90α decapeptide (C10) complex with and without consensus CHIP-binding peptide, and determined the 3.2 Å resolution crystal structure of the human CHIP-C10 decapeptide complex (Task 2.3).

FY03

- Optimized our identified CHIP-binding motif using peptide array technology (Task 2.1).
- Expressed and purified recombinant E2 and E3 to reconstitute an in vitro ubiquitination assay using purified components (Task 2.4).
- Synthesized a DHT-based PROTAC containing our CHIP-targeting motif (Task 2.5).

FY04 (No cost extension)

- Established an in vitro CHIP-dependent ubiquitination assay using AR as substrate (Task 3.1)
- Examined our AR-targeting PROTAC in vitro and in living cells (Task 3.3)

REPORTABLE OUTCOMES

Poster presentation

Tsai, F.T.F., Sielaff, B., Lee, K.S., Smith, D and Kim, K.B. Targeting the Androgen Receptor for Degradation in Prostate Cancer Cells. 2011 IMPaCT Conference. Orlando, Florida (2011).

Smith, D. and Tsai, F.T.F. Harnessing the Ubiquitin-Proteasome System to Develop Novel Drugs. 5th International Conference Sumo, Ubiquitin, UBL Proteins: Implications for Human Diseases. Houston, Texas (2010).

Research presentation (previously reported)

Tsai, F.T.F., Dec. 02, 2008 "Visualizing the ATP-dependent Conformational Changes in a Protein Disaggregating Machine", Department of Molecular and Cellular Biochemistry, University of Kentucky, Lexington, KY. [Purpose: Invited Seminar Speaker and annual task force meeting with Dr. Kyung-Bo Kim, University of Kentucky College of Pharmacy].

Publications (previously reported)

Jang, E.R., Lee, W. and Kim, K.B. (2010). Targeted Degradation of Proteins by PROTACs. *Curr. Protoc. Chem. Biol*. **2**, 71-87.

Cyrus, K., Wehenkel, M., Choi, E.Y., Lee, H., Swanson, H., and Kim, K.B. Jostling for Position: Optimizing Linker Location in the Design of Estrogen Receptor-Targeting PROTACs. *ChemMedChem* 5, 979-985 (2010).
Cyrus, K., Wehenkel, M., Choi, E.Y., Lee, H., Swanson, H., and Kim, K.B. Two-headed PROTAC: An Effective New Tool for Targeted Protein Degradation. *ChemBioChem* 11, 1531-1534 (2010).
Cyrus, K., Wehenkel, M., Choi, E.Y., Han, H.-J., Lee, H., Swanson, H., and Kim, K.B. Impact of linker length on the activity of PROTACs. *Mol. BioSyst.* 7, 359-364 (2011).

List of personnel receiving pay from the research effort
Francis T.F. Tsai, D.Phil. (Principal Investigator)
Bernhard Sielaff, Ph.D. (Baylor College of Medicine, Tsai Lab)
Do-Min Lee, M.S. (University of Kentucky; Kim lab)

CONCLUSIONS

Developing new anti-cancer drugs is imperative to eradicate prostate cancer. PROTAC synthesis represents a powerful approach to target AR for ubiquitination and degradation by the 26S proteasome. Once developed, our PROTACs can be easily modified to target other proteins involved in the development and growth of prostate cancer, respectively.

Progress has been made towards all three tasks outlined in the SoW. During the course of this research, we realized that the E3-substrate relationship is more complex than originally recognized based on our work and that of other groups. For instance, while an E3 recognizes specific proteins as substrate, it is now widely appreciated that the extent and topology of ubiquitination is also dependent on the E2-E3 relationship. While all of our work focused on the CHIP E3 ubiquitin ligase and its major partner E2, Ubc5Hc, there are ~10s of E2s and ~1000 different E3s, which gives rise to a large number of possible E2-E3 combinations. During the course of this work, we used peptide array technology to identify CHIP-binding peptides, which bound CHIP with sub-micromolar affinity. The Arg-Lys-Xxx-Lys-Lys consensus motif is of particular interest as it is found in all known steroid hormone receptors, and was used to generate our first DHT-based PROTAC. To test the efficacy of our PROTAC, we successfully generated bacterially expressed, recombinant human Ubc5Hc and CHIP, which are functional active as determined from our CHIP self-ubiquitination assay. Moreover, we established an in vitro reconstituted CHIP-dependent ubiquitination assay using human Hsp70 as substrate. Notably, Hsp70 is an essential molecular chaperone required for AR folding, and directly relevant to this research. However, we hit a roadblock when using AR as substrate, as no distinct ubiquitinated species could be identified, making it difficult to evaluate whether our DHT-based PROTAC would result in enhanced AR ubiquitination. Similarly, we encountered technical difficulties in targeting AR in our LNCaP cell assay due to an unresponsiveness of the receptor (Prof. K.B. Kim, personal communication). While targeting ER in ER-positive cancer cells was not subject to this technical issue, developing an ER-targeting PROTAC was not previously approved in the SoW and, therefore, not further pursued.

REFERENCES

1. Sakamoto K.M., Kim K.B., Verma R., Ransick A., Stein B., Crews C.M. and Deshaies R.J. Development of Protacs to target cancer-promoting proteins for ubiquitination and degradation. *Mol. Cell. Proteomics* **2**, 1350-1358 (2003).
2. Cyrus K., Wehenkel M., Choi E.Y., Lee H., Swanson H. and Kim K.B. Jostling for position: optimizing linker location in the design of estrogen receptor-targeting PROTACs. *ChemMedChem* **5**, 979-985 (2010).